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<b>(21) International Application Number:</b> PCT/AU99/00968 <b>(22) International Filing Date:</b> 5 November 1999 (05.11.99)  <b>(30) Priority Data:</b> PP 6976 6 November 1998 (06.11.98) AU  <b>(71) Applicant (for all designated States except US):</b> ST. VINCENT'S INSTITUTE OF MEDICAL RESEARCH [AU/AU]; 41 Victoria Parade, Fitzroy, VIC 3065 (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> STAPLETON, David, Ian [AU/AU]; 12 Mint Street, Wantima, VIC 3152 (AU). CHEN, Zhiping [AU/AU]; 14 Eagle Rise, Lower Templestowe, VIC 3107 (AU). MICHELL, Belinda, Joyce [AU/AU]; 12 Rose Avenue, Glen Waverley, VIC 3150 (AU). KEMP, Bruce, Ernest [AU/AU]; 20 Kellett Grove, Kew, VIC 3101 (AU). MITCHELHILL, Kenneth, Ian [AU/AU]; 20 Oxford Street, Mount Waverley, VIC 3149 (AU).  <b>(74) Agent:</b> GRIFFITH HACK; 509 St Kilda Road, Melbourne, VIC 3004 (AU).		<b>(81) Designated States:</b> AU, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY  <b>(57) Abstract</b>  This invention relates to the regulation of the activity of the enzyme nitric oxide synthase, and in particular to regulation of activity of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS and nNOS $\mu$ ). According to a first aspect, the invention provides a method of identifying modulators of AMPK-mediated activation of eNOS, comprising the step of testing putative modulators for their ability to increase or decrease phosphorylation of eNOS depending on the calmodulin and calcium ion concentrations. In an alternative aspect, the invention provides a method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions. Preferably specific phosphorylation of threonine 495 is assessed. According to a second aspect, the invention provides a method of identifying modulators that either promote or inhibit phosphorylation of nNOS and nNOS $\mu$ at Ser-1417. Compounds which activate the AMP-activated protein kinase are expected to be useful in the treatment of ischaemic heart disease by promoting both glucose and fatty acid metabolism, as well as by increasing NOS activity to improve nutrient and oxygen supply to the myocytes and to reduce mechanical activity. These compounds would also have utility in the treatment of pulmonary hypertension and in obstructive airways disease.		

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1                   REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY

2                   This invention relates to the regulation of the  
3                   activity of the enzyme nitric oxide synthase, and in  
4                   particular to regulation of activity of endothelial and  
5                   neuronal nitric oxide synthases. We have found that the  
6                   phosphorylation of endothelial and neuronal nitric oxide  
7                   synthases by several protein kinases, including protein  
8                   kinase C and the AMP-activated protein kinase, regulates  
9                   their activity .

10

11                   BACKGROUND OF THE INVENTION

12                   Nitric oxide (NO) has recently been recognised as  
13                   an important mediator of a very wide variety of cellular  
14                   functions, and is present in most if not all mammalian  
15                   cells (Moncada, S. and Higgs, A., 1993). It is implicated  
16                   in a range of disorders, hypertension,  
17                   hypocholesterolaemia, diabetes, heart failure, aging,  
18                   inflammation, and the effects of cigarette smoking, and is  
19                   especially important in vascular biology. It regulates  
20                   systemic blood pressure as well as vascular remodelling  
21                   (Rudic et al., 1998) and angiogenesis in response to tissue  
22                   ischaemia (Murohara et al., 1998). NO is synthesised from  
23                   the amino acid L-arginine by the enzyme nitric oxide  
24                   synthase (NOS).

25                   Three isoforms of NOS have been identified:  
26                   neuronal NOS (nNOS), which is found in neuronal tissues and  
27                   skeletal muscle (nNOS $\mu$  isoform); inducible NOS (iNOS),  
28                   found in a very wide variety of mammalian tissues including  
29                   activated macrophages, cardiac myocytes, glial cells and  
30                   vascular smooth muscle cells; and endothelial NOS (eNOS),  
31                   found in vascular endothelium, cardiac myocytes and blood  
32                   platelets. Endothelial cells produce NO in response to

1 shear stress generated by the streaming of blood on the  
2 endothelial layer.

3           The three isoforms of NO synthase have an amino  
4 acid sequence identity of approximately 55%, with strong  
5 sequence conservation in regions involved in catalysis.  
6 For all three isoforms, the mechanism of NO synthesis  
7 involves binding of the ubiquitous calcium regulatory  
8 protein calmodulin (CaM) to the enzyme. However, the  
9 conditions under which CaM is bound appear to be different  
10 for iNOS, at least insofar as calcium concentration is  
11 concerned. These three NOS enzymes have been intensively  
12 studied, and the field has been recently reviewed; see for  
13 example Michel and Feron (1997); Harrison (1997); and Mayer  
14 and Hellens (1997). Although it was known from earlier  
15 studies that eNOS could be multiply phosphorylated, the  
16 mechanism of these phosphorylation events, including the  
17 enzyme responsible for phosphorylation, and the role of  
18 phosphorylation in modulation of eNOS function was not  
19 known.

20           AMP-activated protein kinase (AMPK) is a  
21 metabolic stress-sensing protein kinase which is known to  
22 play an important role in the regulation of acetyl-CoA  
23 carboxylase, leading to the acceleration of fatty acid  
24 oxidation during vigorous exercise or ischaemia. AMPK is  
25 well known as a regulator of lipid metabolism, and in  
26 particular is known to have a role in cholesterol  
27 synthesis, as reviewed in Hardie and Carling (1997). The  
28 AMPK is also considered to play an important role in  
29 exercise-enhanced glucose transport (Hayashi et al., (1998)  
30 which is distinct from the insulin-mediated glucose uptake  
31 mechanism. AMPK has mainly been studied in the liver,  
32 heart and skeletal muscle. AMPK has been purified, and the  
33 genes encoding the enzyme subunits were cloned (See  
34 International Patent Applications

1 numbers PCT/GB94/01093 and PCT/US97/00270 and publication  
2 WO97/25341).

3           The mammalian AMPK (Mitchelhill et al, 1994) is  
4 related to the *Saccharomyces cerevisiae* SNF1 protein  
5 kinase. It is required for the expression of glucose-  
6 repressed genes in response to nutritional stress which  
7 requires growth on alternative carbon sources (Celenza and  
8 Carlson, 1986); both the mammalian and yeast kinases are  
9 activated by upstream kinases (Hardie and Carling, 1997).  
10 The AMPK is involved in metabolic stress responses through  
11 phosphorylation at Ser-79 and concomitant inhibition of  
12 acetyl-CoA carboxylase and HMG-CoA reductase (Hardie and  
13 Carling, 1997). Multiple AMPK isoforms occur. They  
14 comprise  $\alpha\beta\gamma$  heterotrimers consisting of either  $\alpha 1$  or  $\alpha 2$   
15 catalytic sub-units (Stapleton et al, 1996; Stapleton et  
16 al, 1997a), together with the non-catalytic subunits  $\beta$  and  
17  $\gamma$  (Mitchelhill et al, 1994; Carling et al, 1994; Stapleton  
18 et al, 1994), which are related to the yeast sip1p and  
19 snf4p respectively.

20           The AMPK  $\alpha 2$  sub-unit gene is on chromosome 1  
21 (Beri et al., 1994), the  $\alpha 1$  sub-unit gene is on  
22 chromosome 5, the  $\beta 1$  and  $\gamma 1$  sub-unit genes are on  
23 chromosome 12, the  $\beta 2$  sub-unit gene is on chromosome 1, and  
24 the  $\gamma 2$  sub-unit gene is localised on chromosome 7  
25 (Stapleton et al, 1997). A  $\gamma 3$  gene has been detected using  
26 an expressed sequence tag (EST) generated by genome  
27 sequencing (Accession No AA178898).

28           One of the genes encoding eNOS is on  
29 chromosome 7, close to the gene for the  $\gamma 2$  sub-unit of  
30 AMPK. Another gene encoding nNOS is found on  
31 chromosome 12. (The human gene map; SEE  
32 <http://www.ncbi.nlm.nih.gov/cgi->

1 bin/SCIENCE96/tsrch?QTEXT=nitric+oxide+synthase)

2           Recent work has shown that the AMPK in cardiac  
3 and skeletal muscle is activated by vigorous exercise or by  
4 ischaemic stress (Winder and Hardie, 1996; Vavvas et al,  
5 1997; Kudo et al, 1995). This led us to investigate the  
6 localization of the AMPK isoforms in these tissues. The  
7 AMPK- $\alpha$ 2 isoform is present in capillary endothelial cells  
8 in cardiac and skeletal muscle, and the AMPK- $\alpha$ 1 isoform  
9 occurs in cardiac myocytes and vessels. The presence of  
10 AMPK in endothelial cells led us to test bacterially-  
11 expressed eNOS as a substrate, and we found that it is  
12 readily phosphorylated by either AMPK- $\alpha$ 1 or AMPK- $\alpha$ 2.

13           We have now surprisingly found that the  
14 AMP-activated protein kinase phosphorylates and regulates  
15 endothelial NO synthase. We find that the AMPK  
16 phosphorylates eNOS at two sites. In the presence of  
17 calcium and calmodulin, Ser-1177 in the human sequence, and  
18 Ser-1179 for the bovine sequence is phosphorylated in the  
19 COOH-terminal tail of the enzyme, causing activation of  
20 eNOS by shifting the calmodulin-dose dependence. In the  
21 absence of added calcium and calmodulin, phosphorylation  
22 also occurs at Thr-495 in the eNOS calmodulin-binding  
23 sequence, and inhibits the enzyme. Ischaemia of the heart  
24 causes activation of the AMPK and of eNOS, mimicking the  
25 effects of phosphorylation at Ser-1177. Phosphopeptide-  
26 specific antibodies to phosphorylated Ser-1177 were used to  
27 confirm that this site was phosphorylated during ischaemia.  
28 Our results are of special interest because they identify a  
29 link between metabolic stress, which reduces ATP and  
30 increases AMP, and signalling through eNOS to control  
31 nutrient availability (via arterial vasodilation) as well  
32 as suppressing myocardial contraction. This couples the  
33 metabolic status of endothelial cells and myocytes with the

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1 vascular supply and mechanical demands. Our results  
2 provide a new insight into the post-translational  
3 regulation of eNOS which is of particular significance for  
4 the cardiovascular and skeletal muscle field. In addition,  
5 similarities in structure and behaviour between eNOS and  
6 nNOS have been identified, enabling us to identify  
7 modulators of the activity of both these enzymes.

8

9 SUMMARY OF THE INVENTION

10 According to a first aspect, the invention  
11 provides a method of identifying modulators of AMPK-  
12 mediated activation of a nitric oxide synthase enzyme  
13 selected from the group consisting of eNOS, nNOS and nNOS $\mu$ ,  
14 comprising the step of testing the ability of putative  
15 modulators to increase or decrease phosphorylation of the  
16 enzyme; said increase or decrease depending on the  
17 calmodulin and calcium ion concentrations.

18 Preferably the specific phosphorylation of  
19 Ser-1177 is assessed in the presence of calcium and  
20 calmodulin.

21 In an alternative aspect, the invention provides  
22 a method of identifying modulators of AMPK-mediated  
23 inhibition of eNOS, comprising the step of testing a  
24 putative modulator for its ability to decrease or increase  
25 AMPK-mediated phosphorylation of eNOS in the presence of  
26 limiting calcium ions. Preferably specific phosphorylation  
27 of Thr-495 is assessed.

28 Compounds able to increase phosphorylation of  
29 Ser-1177 or decrease phosphorylation of Thr-495 are  
30 referred to herein as activators, and compounds able to  
31 decrease phosphorylation of Ser-1177 or increase  
32 phosphorylation of Thr-495 are referred to as inhibitors.

1           In both aspects of the invention, one or more of  
2   the following activities may optionally be additionally  
3   assessed for each putative activator or inhibitor  
4   identified by the method of the invention:

- 5       (a)   Effect on smooth muscle contraction;
- 6       (b)               Effect on inotropic activity of the  
7                       heart;
- 8       (c)               Effect on chronotropic activity of the  
9                       heart; and
- 10      (d)               Effect on platelet function.

11           It is expected that because the phosphorylation  
12   site equivalent to Thr-495 in the eNOS calmodulin-binding  
13   site is absent from the neuronal form of NOS, inhibitors  
14   and activators identified by the method of the invention  
15   will have at least some degree of tissue specificity.

16           Compounds that activate the AMP-activated protein  
17   kinase are expected to be useful in ischaemic heart disease  
18   by promoting both glucose and fatty acid metabolism, as  
19   well as by increasing NOS activity to improve nutrient and  
20   oxygen supply to the myocytes and to reduce mechanical  
21   activity. These compounds would also have utility in  
22   pulmonary hypertension and in obstructive airways disease.

23           For the purposes of this specification it will be  
24   clearly understood that the word "comprising" means  
25   "including but not limited to", and that the word  
26   "comprises" has a corresponding meaning.

27

#### 28   BRIEF DESCRIPTION OF THE FIGURES

29           Figure 1 shows immunofluorescence localization of  
30   AMPK- $\alpha$ 2 in the heart and in the tibialis anterior muscle.



1 Panel A shows a negative control section of rat  
2 heart stained with control rabbit IgG and control mouse  
3 IgG, together with anti-rabbit-FITC and anti-mouse-Texas  
4 Red.

5 Panel B shows a section of rat heart stained with  
6 affinity-purified rabbit polyclonal antibody against  
7 AMPK- $\alpha$ 2 (491-514) and anti-rabbit-FITC.

8 Panel C shows the same section as Panel B,  
9 stained with a monoclonal antibody against rat endothelium  
10 recA-1 and anti-mouse-Texas Red.

11 Panel D shows the overlay of Panels B and C.  
12 Colocalization can be seen by the coincidence of staining.  
13 The arrows highlight specific endothelial cells that are  
14 stained by both antibodies.

15 Panel E shows a negative control section of rat  
16 tibialis anterior muscle stained with control rabbit IgG  
17 and control mouse-IgG, together with anti-rabbit-FITC and  
18 anti-mouse-Texas Red.

19 Panel F shows a section stained with affinity-  
20 purified rabbit polyclonal antibody against AMPK- $\alpha$ 2  
21 (491-514) and anti-rabbit-FITC.

22 Panel G shows the same section as in Panel B,  
23 stained with a monoclonal antibody against rat endothelium  
24 recA-1 and anti-mouse-Texas Red.

25 Panel H shows the overlay of Panels E and F.  
26 Colocalization can be seen by the coincidence of staining.

27 Figure 2 illustrates phosphorylation of  
28 recombinant eNOS by AMPK.

29 Top panel: eNOS was incubated with rat liver  
30 AMPK- $\alpha$ 1 and [ $\gamma$ - $^{32}$ P] ATP.

31 Lane 1: Coomassie-stained SDS-PAGE;

1 Lane 2: Autoradiograph.

2 Lower panel:  $^{32}\text{P}$ -tryptic phosphopeptide map of  
3 eNOS.

4 Figure 3 shows the effect of phosphorylation of  
5 eNOS by the AMPK with or without added  $\text{Ca}^{2+}$ -CaM. Rat  
6 heart eNOS purified by 2',5'-ADP-Sepharose affinity  
7 chromatography was phosphorylated by AMPK in the presence  
8 of  $0.8\ \mu\text{M}$  CaM/ $3.2\ \mu\text{M}$   $\text{Ca}^{2+}$  (closed circles), in the absence  
9 of  $\text{Ca}^{2+}$ -CaM (closed triangles) and without AMPK (open  
10 squares). After phosphorylation, samples were diluted and  
11 eNOS activity was measured. The lower panels show  
12 phosphopeptide maps for rat heart eNOS phosphorylated in  
13 the presence and absence of added  $\text{Ca}^{2+}$ -CaM.

14 Figure 4 shows the effect of ischaemia on the  
15 activities of AMPK- $\alpha 1$ , AMPK- $\alpha 2$  and eNOS.

16 Panel A shows the results of immunoprecipitation  
17 using antibody specific for AMPK- $\alpha 1$  and AMPK- $\alpha 2$ , assayed  
18 using the SAMS peptide substrate. Results shown are mean  $\pm$   
19 SEM for  $n=5$ .

20 Panel B shows eNOS activity measured at  $500\ \text{nM}$   
21 CaM.

22 Panel C shows eNOS activities with full CaM-dose  
23 responses for a representative experiment. Ischaemia time  
24 points: 0 min (open squares), 1 min (closed diamonds),  
25 10 min (closed circles) and 20 min (open triangles). The  
26 results of 4 replicates were the same, except that in one  
27 case the 20 min ischaemia eNOS CaM-dependence remained the  
28 same as for 10 min.

29 Figure 5 shows a comparison of NOS sequences.  
30 Phosphorylation site sequences for eNOS and nNOS are  
31 indicated in a schematic model of NOS. Sequences from the  
32 CaM-binding region (around the Thr-495 phosphorylation site

- 9 -

1 in eNOS) and for the COOH-terminal tail (around the  
2 Ser-1177 phosphorylation site in eNOS) are shown.

3 Figure 6 shows the effect of treatment of bovine  
4 aortic endothelial cells with phorbol ester (PMA) and  
5 okadaic acid on eNOS activity (upper pane) and the  
6 phosphorylation at Ser-1177 and Thr-495 (lower panel).

7 Figure 7 shows the effect of treatment of bovine  
8 aortic endothelial cells with 3-isobutyl-1-methylxanthine  
9 (IBMX) and calyculin A on the phosphorylation at Ser-1177  
10 and Thr-495.

11 Figure 8 shows a summary illustration of the  
12 regulation of eNOS by phosphorylation at Thr-495 and Ser-  
13 1177, mediated by protein kinases PKC, AMPK and Akt.  
14 Reversal of the phosphorylation at these sites is mediated  
15 by protein phosphatases PP1 and PP2A in response to  
16 treating the cells with IBMX and PMA respectively.

17 Figure 9 shows the effect of a 30 second bicycle  
18 sprint exercise on nNOS phosphorylation in human muscle.  
19 The nNOS was extracted from biopsy material and probed for  
20 phosphorylation at Ser-1417 using an anti-phosphopeptide  
21 antibody. The left panel shows an immunoblot, and the  
22 right panel shows quantitative analysis of 5 individuals.

23

#### 24 DETAILED DESCRIPTION OF THE INVENTION

25 The invention will now be described in detail by  
26 way of reference only to the following non-limiting  
27 examples and to the figures.

28 We have surprisingly found that in the presence  
29 of  $\text{Ca}^{2+}$ -calmodulin (CaM) eNOS is phosphorylated by AMPK at  
30 Ser-1177, resulting in activation, whereas phosphorylation  
31 of eNOS in the absence of  $\text{Ca}^{2+}$  occurs predominantly at  
32 Thr-495, a site in the CaM-binding sequence, resulting in

1 inhibition. It had previously been considered that  
2 phosphorylation was solely inhibitory. We have also found  
3 that ischaemia of the heart leads to rapid activation of  
4 both isoforms of the metabolic stress-sensing enzyme AMPK  
5 and eNOS. These data suggest that the AMPK may operate an  
6 "inside-out" signalling pathway that leads to arterial  
7 vasodilation and reduced myocardial contraction, so  
8 coupling the metabolic status of endothelial cells and  
9 myocytes with the vascular supply and mechanical activity.

10

11 Example 1                    Immunofluorescence Localisation of AMPK- $\alpha$ 2  
12                                    in Heart and Skeletal Muscle

13                    Confocal immunofluorescence microscopy using  
14 affinity-purified rabbit polyclonal antibody directed  
15 against AMPK- $\alpha$ 2 (antibody 491-414. Staining with  
16 fluorescence-labelled anti-rabbit antibody showed that the  
17  $\alpha$ 2 isoform is found predominantly in capillary endothelial  
18 cells in both cardiac muscle and skeletal muscle, while  
19 cardiac myocytes and blood vessels showed intense but  
20 diffuse staining for the  $\alpha$ 1 AMPK isoform. In skeletal  
21 muscle, the  $\alpha$ 2 isoform was found in endothelial cells of  
22 capillaries, and in fast-twitch muscle fibres, whereas the  
23  $\alpha$ 1 isoform was found in Type I aerobic fibres.

24 Localisation of AMPK- $\alpha$ 2 in capillary endothelial cells in  
25 both cardiac and skeletal muscle is illustrated in  
26 Figure 1.

27

28 Example 2                    AMPK Phosphorylates Recombinant eNOS

29                    Bacterially expressed eNOS, coexpressed with CaM  
30 by the method of Rodriguez-Crespo et al (1996), was  
31 phosphorylated by either AMPK- $\alpha$ 1, as shown in Figure 2 top

1 panel, or AMPK- $\alpha$ 2. Recombinant eNOS phosphorylation by  
2 immunoprecipitated AMPK- $\alpha$ 2 was detected. Since we have  
3 been unable to purify high specific activity AMPK- $\alpha$ 2, no  
4 further characterisation of eNOS regulation or the sites of  
5 phosphorylation by the  $\alpha$ 2 isoform was undertaken. Analysis  
6 of the phosphorylation sites in eNOS following tryptic  
7 digestion revealed four phosphopeptides generated from  
8 three separate sites (Figure 2 bottom panel, A, A', B, C).  
9 Identification of phosphorylation sites by mass  
10 spectrometry and Edman sequencing, using the modified  
11 method described by (Mitchelhill and Kemp, 1999), revealed  
12 that Ser-1177 was the most prominent phosphorylation site,  
13 as shown in Figure 2 bottom panel, A, A', and that its  
14 phosphorylation was dependent on the presence of  $\text{Ca}^{2+}$ -CaM.

15           Phosphopeptide isolation from in-gel tryptic  
16 digests was carried out as described by Mitchelhill et al  
17 (1997a). Greater than 98% of the radioactivity was  
18 recovered from the gel. Peptides isolated and characterized  
19 by mass spectrometry and Edman sequencing are set out in  
20 Table 1.

Table 1  
Phosphopeptides Isolated from In-Gel tryptic Digests

Observed Mass	Phosphopeptide	Sequence	Calculated Mass
1440.0	B	KKTFKEVANAVK	1361.1 (*1441.7)
1174.1	A	TQXFSLQER	1094.5 (*1174.5)
1445.6	A'	IRTQXFSLQER	1363.7 (*1443.7)
1176.7	C	pcLGSLVFPR	1095.6 (*1175.6)

10

where: "pc" denotes pyridylethyl cysteine.

• denotes calculated mass of mono-phosphorylated peptide.

15

1           The location of the phosphorylation site in  
2 peptide A, TQXFSLQER, was identified by  $^{32}\text{P}$ -phosphate  
3 release sequencing (Mitchelhill et al, 1997a). eNOS  
4 phosphorylated by the AMPK- $\alpha$ 1 was no longer recognized by  
5 the antibody to the eNOS COOH-terminal tail; nor was it  
6 eluted from the ADP-Sepharose affinity column by  
7 100 mM NADPH. These properties prevented the direct  
8 confirmation of Ser-1177 phosphorylation *in situ*. This is  
9 illustrated in Venema et al, 1996.

10           A second site, Thr-495, was phosphorylated in the  
11 absence of  $\text{Ca}^{2+}$ -CaM or when EGTA was present. This is  
12 illustrated in Figure 2 bottom panel, B. This residue is  
13 located in the CaM-binding sequence,

14                           TRKKT<sup>495</sup>FKEVANAVKISASLM,

15 between the oxidase and reductase domains of eNOS (Venema  
16 et al, 1996). Ser-101 in the N-terminal region of eNOS was  
17 identified as a minor site of phosphorylation (Figure 2  
18 bottom panel, C).

19           Synthetic peptides containing Thr-495 or Ser-1177  
20 were readily phosphorylated by AMPK, with similar kinetic  
21 values to the SAMS peptide substrate. The peptide  
22 containing Thr-495, GTGITRKKTFKEVANAVK, was phosphorylated  
23 with a  $K_m$  of  $39 \pm 10 \mu\text{M}$  and a  $V_{max}$  of  
24  $6.7 \pm 0.6 \mu\text{mol/min/mg}$ , whereas the peptide containing  
25 Ser-1177, RIRTQSFSLQERQLRG was phosphorylated with a  $K_m$  of  
26  $54 \pm 6 \mu\text{M}$  and a  $V_{max}$  of  $5.8 \pm 0.3 \mu\text{mol/min/mg}$ . These are  
27 comparable to results obtained using the well-characterized  
28 SAMS peptide substrate, which has a  $K_m$   $33 \pm 3 \mu\text{M}$  and a  $V_{max}$   
29 of  $8.1 \pm 1.5 \mu\text{mol/min/mg}$  (Michell et al, 1996). The *in*  
30 *vitro* phosphorylation of the peptides confirms the  
31 identification sites of phosphorylation.

1    Example 3                    Effect of Ca<sup>2+</sup>-CaM on Phosphorylation of  
2                                    eNOS by AMPK

3                    The eNOS activity was determined by measuring  
4    L-[<sup>3</sup>H]-citrulline production, using the method of Balligand  
5    et al, 1995. The recombinant eNOS was coexpressed with  
6    CaM, as described by Rodriguez-Crespo and Ortiz de  
7    Montellano, 1996. Partially purified rat heart eNOS  
8    contained some Ca<sup>2+</sup>-CaM. In the absence of added EGTA, CaM  
9    dependence was observed at 0-100 nM added CaM. In order to  
10   investigate the changes in NOS activity with  
11   phosphorylation in the absence and presence of Ca<sup>2+</sup>-CaM,  
12   EGTA buffering was used to achieve CaM dose response curves  
13   in the range 0-1 µM. Routinely, 7-15 µM EGTA was added to  
14   make eNOS activity dependent upon added CaM. Where  
15   Ca<sup>2+</sup>-CaM was used in the phosphorylation reaction prior to  
16   eNOS assay, the samples were either diluted so that the  
17   extra Ca<sup>2+</sup>-CaM was negligible, or the indicated  
18   concentrations represent total final concentrations of  
19   added Ca<sup>2+</sup>-CaM.

20                    Cardiac eNOS was partially purified as follows.  
21   Twenty rat hearts were homogenised in 80 ml of ice-cold  
22   buffer A [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA,  
23   1 mM DTT, 50 mM NaF, 5 mM Na Pyrophosphate,  
24   10 µg/ml Trypsin inhibitor, 2 µg/ml Aprotinin,  
25   1 mM Benzamidine, 1 mM PMSF, 10% Glycerol, 1% Triton-X-  
26   100]. The homogenate was put on ice for 30 min and  
27   centrifuged at 16,000 x g for 30 min. The supernatant was  
28   incubated with 2 ml of 2',5'-ADP-Sepharose (Bredt and  
29   Snyder, 1990). The suspension was incubated for one hour  
30   before washing in a fritted column, with 20 ml of buffer A  
31   and 20 ml of buffer A containing 0.5 M NaCl, and then with  
32   20 ml of buffer B [50 mM Tris-HCl, pH 7.5, 1 mM DTT,  
33   10% Glycerol, 0.1% Triton-X-100]. eNOS was eluted with  
34   buffer B containing 2 mM NADPH, then subjected to



1 centrifugal filtration (ULTRAFREE-MC MILLIPORE) to remove  
2 NADPH. Immunoblotting was used for selective detection of  
3 eNOS rather than nNOS.

4           Phosphorylation of eNOS by AMPK in the presence  
5 of  $\text{Ca}^{2+}$ -CaM resulted in activation, but CaM-dependence was  
6 retained, as shown in Figure 3 top panel. Activation  
7 shifted the dose response curve for CaM to the left.  
8 Phosphopeptide mapping revealed that activation of eNOS was  
9 correlated with phosphorylation of Ser-1177 but not of Thr-  
10 495, as shown in Figure 3 lower panel. Phosphorylation  
11 without added  $\text{Ca}^{2+}$ -CaM enhanced Thr-495 phosphorylation,  
12 suppressed Ser-1177 phosphorylation, and inhibited eNOS  
13 activity (Figure 3 top panel). The inhibition of eNOS  
14 activity by Thr-495 phosphorylation is consistent with  
15 earlier reports that phosphorylation of synthetic peptides  
16 corresponding to this region by protein kinase C inhibits  
17 CaM-binding (Matsubara et al, 1996). Similar results have  
18 been reported for nNOS (Loche et al, 1997).

19

20 Example 4           Effect of Ischaemia on Activities of  
21                           AMPK- $\alpha$ 1, AMPK- $\alpha$ 2 and eNOS

22           Langendorf preparations of isolated perfused rat  
23 heart were subjected to ischaemia according to the method  
24 of Kudo et al (1995). AMPK- $\alpha$ 1 and AMPK- $\alpha$ 2 isoforms were  
25 immunoprecipitated using  $\alpha$ 2 (490-516) or  $\alpha$ 1 (231-251)  
26 antibodies, and assayed using the SAMS peptide substrate  
27 (Michell et al, 1996; Hardie and Carling, 1997). eNOS  
28 activity was measured as described in Example 3. The  
29 results are shown in Figure 4. Both  $\alpha$ 1 and  $\alpha$ 2 isoforms are  
30 activated, as shown in Figure 4A, indicating that AMPK is  
31 activated in both capillary endothelial cells, which have  
32 predominantly the  $\alpha$ 2 isoform, and in cardiac myocytes,

1 which have predominantly the  $\alpha 1$  isoform. AMPK activation  
2 during ischaemia is also accompanied by eNOS activation and  
3 changes in the CaM dependence, as shown in Figures 4B and  
4 4C, mimicking the effect of eNOS phosphorylation by AMPK *in*  
5 *vitro*, as shown in Figure 3.

6 Polyclonal antibodies were raised against  
7 synthetic phosphopeptides based on the eNOS sequence:  
8 RIRTQSpFSLQER and GITRKKTpFKEVANCV. Rabbits were immunized  
9 with phosphopeptides coupled to keyhole limpet haemocyanin  
10 and then emulsified in Freund's complete adjuvant, using  
11 conventional methods. The antibodies were purified using  
12 the corresponding phosphopeptide affinity columns after  
13 thorough preclearing with dephosphopeptide affinity  
14 columns. The specificity of the purified antibodies was  
15 confirmed using both EIA and immunoblotting, confirming  
16 that they did not recognize recombinant dephospho-eNOS.

17 Using the anti-phosphopeptide antibodies to Ser-  
18 1177 and Thr-495 phosphorylation sites we observed that  
19 phosphorylation of Ser-1177 was increased approximately 3-  
20 fold by ischaemia, but that there was no detectable change  
21 in the Thr-495 phosphorylation under these conditions.  
22 Heart muscle contains eNOS in both capillary endothelial  
23 cells and cardiac myocytes (Balligand *et al*, 1995), with  
24 low levels of the nNOS  $\mu$  isoform (Silvagno *et al*, 1996).

25 The sequences of the three types of NOS are  
26 compared in Figure 5, which shows the CaM-binding region  
27 and the C-terminal tail. In nNOS Ser-1417 corresponds to  
28 eNOS Ser-1177, whereas iNOS is truncated, and has a Glu in  
29 this region. Both iNOS and nNOS lack a phosphorylatable  
30 residue equivalent to Thr-495 in the CaM-binding region.

Example 5      Effect of Stimulation of Protein Kinase C on  
eNOS Phosphorylation

Bovine aortic endothelial cells cultured in 0.1% foetal calf serum for 20 hours (serum starved) were subjected to treatment with the protein kinase C activator 0.1  $\mu$ M phorbol-12-myristate-13-acetate (PMA) for 5 min. PMA treatment increased the phosphorylation of eNOS at Thr-495 and decreased the phosphorylation at Ser-1177, as measured using anti-phosphopeptide specific antibodies. The antibodies used were the same as those described in Example 4. The results are shown in Figure 6. In cells cultured in medium without calcium we observed a 4-fold decrease in Ser-1177 phosphorylation. Furthermore, when cells were incubated in standard medium containing calcium addition of the calcium ionophore A23187 (10 $\mu$ M for 90 seconds) increased Ser-1177 phosphorylation by a further 7-fold. Preincubation of the cells with 0.5  $\mu$ M okadaic acid prevented the dephosphorylation of Ser-1177 by PMA treatment, and greatly augmented the phosphorylation of Thr-495 (Results mean  $\pm$  SEM, n =6). Since okadaic acid inhibits protein phosphatase PP2A, the results indicate that PP2A is responsible for dephosphorylation of Ser-1177. The changes observed in Thr-495 and Ser-1177 phosphorylation in response to treatment with PMA and okadaic acid were reflected in the activity of eNOS. Increased phosphorylation of Thr-495 with PMA or PMA plus okadaic acid was associated with reduced eNOS activity. Okadaic acid alone increased Ser-1177 phosphorylation without altering Thr-495 phosphorylation, and was associated with increased eNOS activity (Figure 6 upper panel).

Example 6            Effect of Inhibition of Phosphodiesterase  
and Phosphatase on the Phosphorylation of eNOS

The experimental details were similar to those for Example 5. Bovine aortic endothelial cells were preincubated with or without 10 nM of the phosphatase inhibitor calyculin A for 10 min, and then incubated with or without 0.5 mM of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) for 5 min. As shown in Figure 7, IBMX treatment caused enhanced phosphorylation of Ser-1177 and dephosphorylation of Thr-495. Preincubation with calyculin A prevented the dephosphorylation of Thr-495. (Results mean  $\pm$  SEM, n =6). Since calyculin A inhibits protein phosphatase PP1, the results indicate that PP1 is responsible for dephosphorylation of Thr-495.

DISCUSSION

Since the identification of the Ser-1177 phosphorylation site by the present inventors, it has been recognized that other protein kinases phosphorylate at this site. In particular, the protein kinase Akt (also named PKB) phosphorylates Ser-1177 in response to stimulation of endothelial cells by vascular endothelial growth factor (VEGF) (Fulton et al.1999; Michell et al.,1999) or to fluid shear stress (Dimmeler et al., 1999; Gallis et al., 1999). In the study by Gallis et al. (1999) it was reported that fluid shear stress stimulated the phosphorylation of Ser-116 in the sequence KLQTRPSPGPPPA. Neither the kinase responsible nor the functional effects of phosphorylation of this site on eNOS has yet been identified. This phosphorylation site is present in the oxidase domain.

1                   We have found that phosphorylation of eNOS at  
2 Thr-495 by protein kinase C occurs in endothelial cells  
3 that have been serum starved and incubated in calcium-free  
4 medium in the presence of the phorbol ester PMA. There is  
5 a reciprocal relationship between phosphorylation at Ser-  
6 1177 and Thr-495 in endothelial cells. Protein kinase C  
7 phosphorylates both sites *in vitro*, but stimulation of  
8 protein kinase C in endothelial cells with phorbol ester  
9 causes enhanced Thr-495 phosphorylation but marked  
10 phosphorylation of Ser-1177. The dephosphorylation of Ser-  
11 1177 is prevented by okadaic acid but not by calyculin A,  
12 indicating that phosphatase PP2A is responsible. Okadaic  
13 acid also greatly enhances the phosphorylation of Thr-495  
14 in response to phorbol ester. Thrombin, which also acts  
15 via protein kinase C, stimulates phosphorylation of Thr-495  
16 and dephosphorylation of Ser-1177.

17                   In contrast, treatment of endothelial cells with  
18 the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine  
19 (IBMX) causes a pronounced dephosphorylation of eNOS at  
20 Thr-495 and enhanced Ser-1177 phosphorylation.  
21 Dephosphorylation of Thr-495 in response to IBMX is blocked  
22 by treatment with calyculin A, suggesting that phosphatase  
23 PP1 is responsible for Thr-495 dephosphorylation.

24                   These relationships are summarised in Figure 8.  
25 We find that exercise of skeletal muscle results in the  
26 phosphorylation of nNOS $\mu$  at Ser-1417, the site  
27 corresponding to Ser-1177 in eNOS (see Figure 5).  
28 Electrical stimulation of rat extensor digitorum longus  
29 (EDL) muscle was found to activate the AMPK, to  
30 phosphorylate acetyl CoA carboxylase at Ser-79 (the  
31 inhibitory site), and to phosphorylate nNOS $\mu$  at Ser 1417.  
32 Similarly, in biopsies of human skeletal muscle following  
33 vigorous exercise, such as a 30-second bicycle sprint,  
34 there is a 10-fold increase in phosphorylation on Ser-79 in

1 acetyl CoA carboxylase and a 7.5-fold increase in nNOS $\mu$   
2 phosphorylation at Ser-1417 (see Figure 9).

3           Endothelially-derived NO has a critical role in  
4 preventing premature platelet adhesion and aggregation that  
5 leads to thrombus formation (Radomski and Moncada, 1993).  
6 There is evidence that the protective effects of elevated  
7 high-density lipoprotein (HDL) on the cardiovascular system  
8 may be mediated via increased platelet NO production.  
9 Apolipoprotein E, a component of HDL, acts on a receptor  
10 (apoER2) present in platelets to stimulate the NO signal  
11 transduction pathway (Riddell et al., 1997; Riddell and  
12 Owen, 1999).

13           Activation of eNOS by phosphorylation of its  
14 COOH-terminal tail gives new insight into eNOS  
15 autoinhibition. The increased activity and shift in the  
16 CaM-dose dependence with phosphorylation at Ser-1177  
17 suggest that in eNOS, and perhaps nNOS, the COOH-terminal  
18 tails act as partial autoregulatory sequences analogous to  
19 those in the CaM-dependent protein kinases (Kemp and  
20 Pearson, 1991; Kobe et al, 1996).

21           The COOH-terminal tail of eNOS is only fully  
22 accessible to the AMPK when Ca<sup>2+</sup>-CaM is bound, consistent  
23 with this region being buried in the absence of CaM. As  
24 can be seen from Figure 5, there is a high level of  
25 similarity between eNOS and nNOS in their COOH-terminal  
26 tails, whereas iNOS is distinct. It is known that the iNOS  
27 CaM-binding, which is characterised by a low  
28 Ca<sup>2+</sup>-dependence, requires both the canonical CaM-binding  
29 sequence and distal residues in the COOH-terminus that  
30 cannot be satisfied by nNOS chimeras (Ruan et al, 1996).  
31 Without wishing to be bound by any proposed mechanism, we  
32 believe that eNOS and nNOS are autoinhibited by their  
33 COOH-terminal tails, requiring a two-stage activation

1 process for full activity with both CaM-binding and  
2 phosphorylation in the tail, whereas iNOS requires only CaM  
3 binding. Recently Salerno et al (1997) proposed that an  
4 insert sequence in the FMN-binding domain may also be  
5 important in autoregulation.

6 Previous studies have shown that eNOS may be  
7 phosphorylated both *in vitro* and *in vivo*, but the precise  
8 sites of phosphorylation and the function of the  
9 phosphorylation events have not hitherto been fully  
10 characterized (reviewed in Michel and Feron, 1997). eNOS  
11 is the first example of an enzyme activated by AMPK to be  
12 identified, and is also unusual because phosphorylation can  
13 lead to either activation or inhibition, depending on the  
14 availability of  $\text{Ca}^{2+}$ -CaM. Other enzymes, notably the  
15 cyclin-dependent protein kinases, are activated or  
16 inhibited by phosphorylation, but this is catalysed by  
17 different protein kinases. Protein kinase C phosphorylates  
18 Thr-495 in eNOS, demonstrating intersecting regulatory  
19 pathways acting on eNOS by phosphorylation of Thr-495 or  
20 Ser-1177. It is also possible that persistent activation  
21 of protein kinase C, for example in response to  
22 hyperglycaemia induced by diabetes, could chronically  
23 suppress phosphorylation of eNOS at Ser-1177, and thereby  
24 reduce its activity.

25 The regulation of eNOS by AMPK extends the  
26 conceptual relationship between the yeast snflp kinase and  
27 the AMPK. Snflp kinase modulates the supply of glucose from  
28 the environment by secreting invertase whereas the  
29 mammalian AMPK integrates metabolic stress signalling with  
30 the control of the circulatory system. Thus intracellular  
31 metabolic stress signals within endothelial cells and  
32 myocytes can elicit improved nutrient supply and suppress  
33 mechanical activity of the muscle.

1           It will be apparent to the person skilled in the  
2 art that while the invention has been described in some  
3 detail for the purposes of clarity and understanding,  
4 various modifications and alterations to the embodiments  
5 and methods described herein may be made without departing  
6 from the scope of the inventive concept disclosed in this  
7 specification.

8           References cited herein are listed on the  
9 following pages, and are incorporated herein by this  
10 reference.



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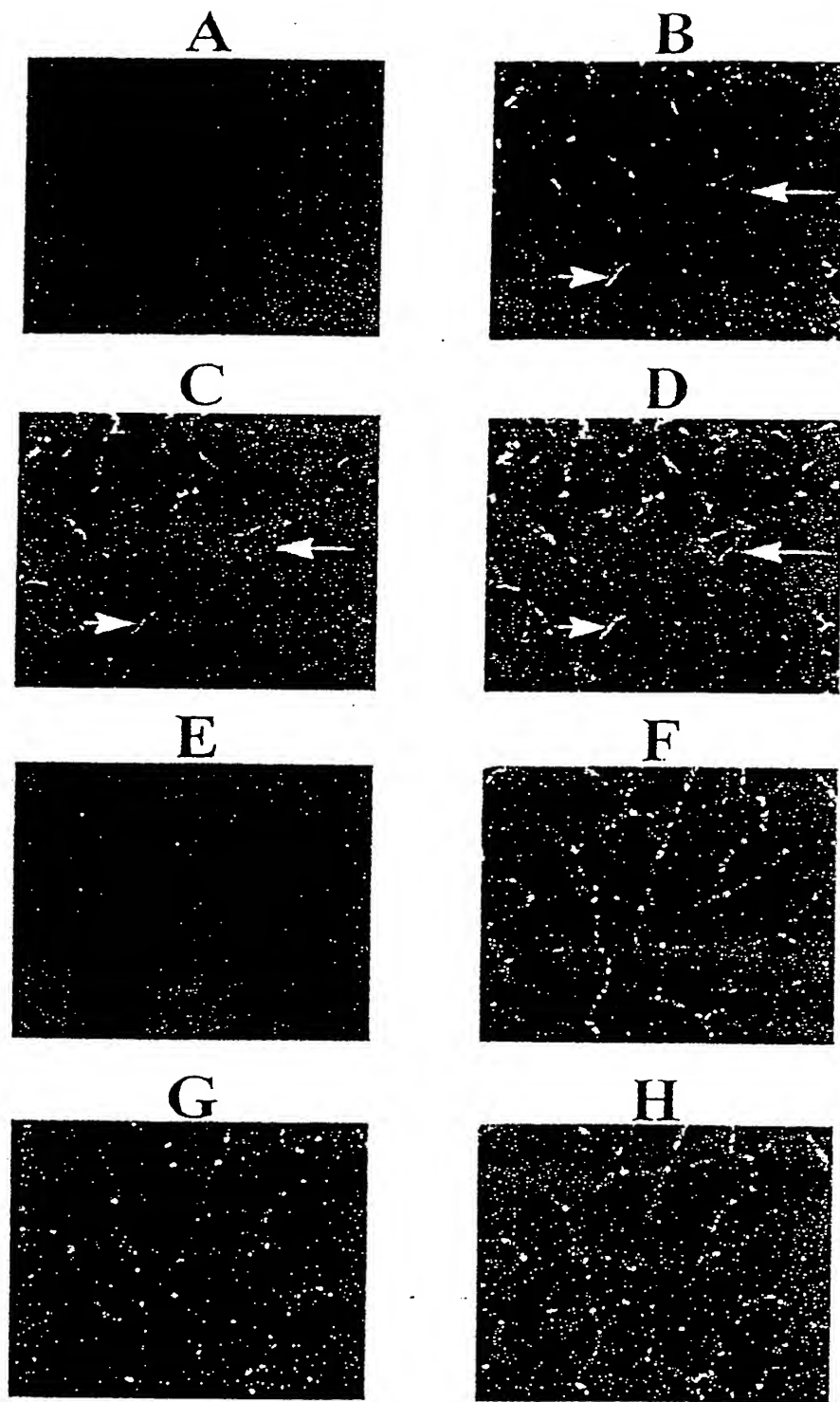
CLAIMS:

1. A method of identifying modulators of AMPK-mediated activation of a nitric oxide synthase enzyme selected from the group consisting of eNOS, nNOS and nNOS $\mu$ , comprising  
5 the step of testing putative modulators for their ability to increase or decrease phosphorylation of the enzyme, said increase or decrease depending on the calmodulin and calcium ion concentrations.
2. A method according to claim 1, in which the specific  
10 phosphorylation of Ser-1177 is assessed in the presence of calcium and calmodulin.
3. A method of identifying modulators of AMPK-mediated inhibition of eNOS, comprising the step of testing a  
15 putative modulator for its ability to decrease or increase AMPK-mediated phosphorylation of eNOS in the presence of limiting calcium ions.
4. A method according to claim 3, in which the specific phosphorylation of Thr-495 is assessed.
5. A method according to any one of Claims 1 to 4, in  
20 which one or more of the following activities is additionally assessed:
  - (a) Effect on smooth muscle contraction;
  - (b) Effect on inotropic activity of the heart;
  - (c) Effect on chronotropic activity of the heart; or
  - 25 (d) Effect on platelet function.
6. A method according to any one of Claims 1 to 5, in which the modulator is an activator, as herein defined.
7. A method according to Claim 6, in which the activator promotes both glucose metabolism and fatty acid metabolism.

- 29 -

8. A method according to any one of Claims 1 to 5, in which the modulator is an inhibitor, as herein defined.
9. A method according to any one of Claims 3 to 8, in which the modulator acts preferentially on non-neuronal  
5 cells.
10. A method according to Claim 1 or Claim 2, in which the modulator promotes the dephosphorylation of Ser-1177 and inhibits eNOS activity.
11. A method according to Claim 3, in which the modulator  
10 promotes the dephosphorylation of Thr-495 and stimulates eNOS activity.
12. A method according to Claim 1 or Claim 2 in which the modulator promotes phosphorylation of nNOS or nNOS $\mu$  at Ser-1417.
- 15 13. A method according to Claim 1 or Claim 2 in which the modulator promotes dephosphorylation of nNOS or nNOS $\mu$  at Ser-1417.

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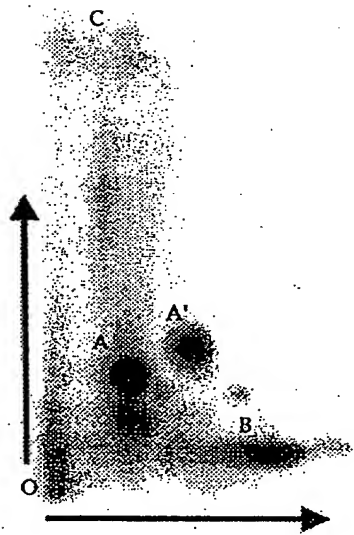
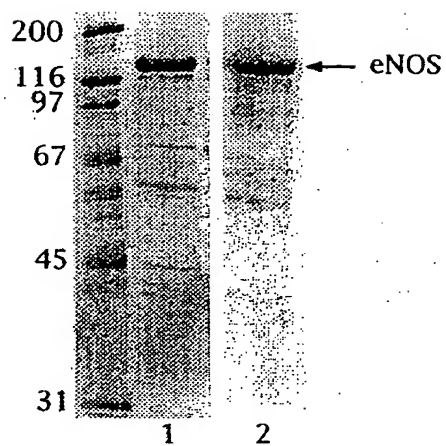


*Figure 1*

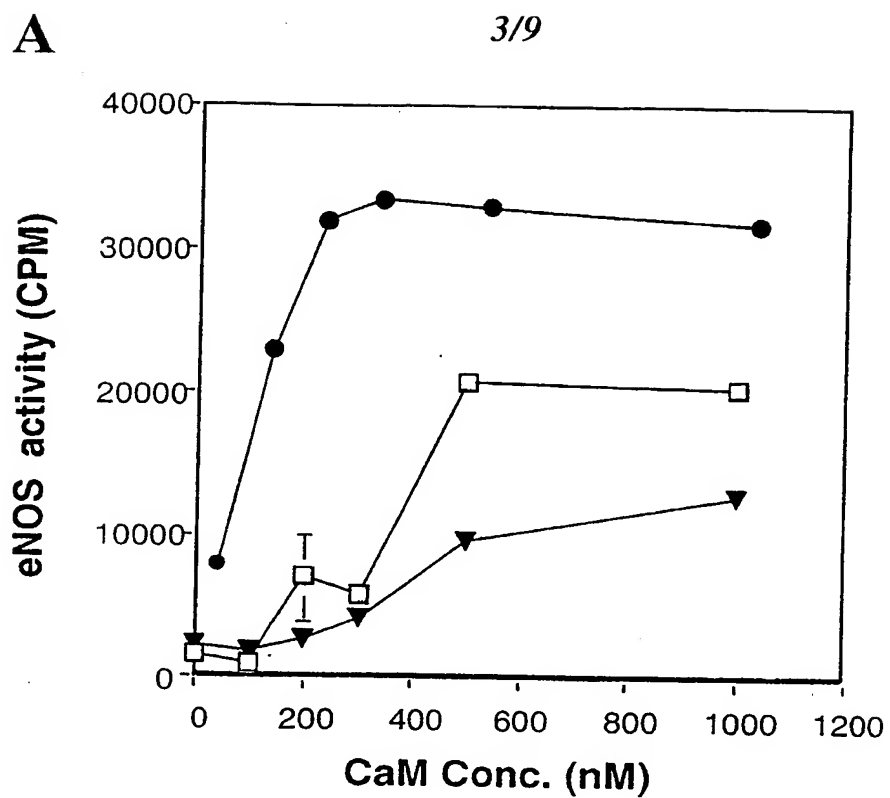
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*Figure 2*

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eNOS + AMPK  
+ Ca<sup>2+</sup>-CaM

eNOS + AMPK

**A** **A** **B**

**O** **O**

*Figure 3*

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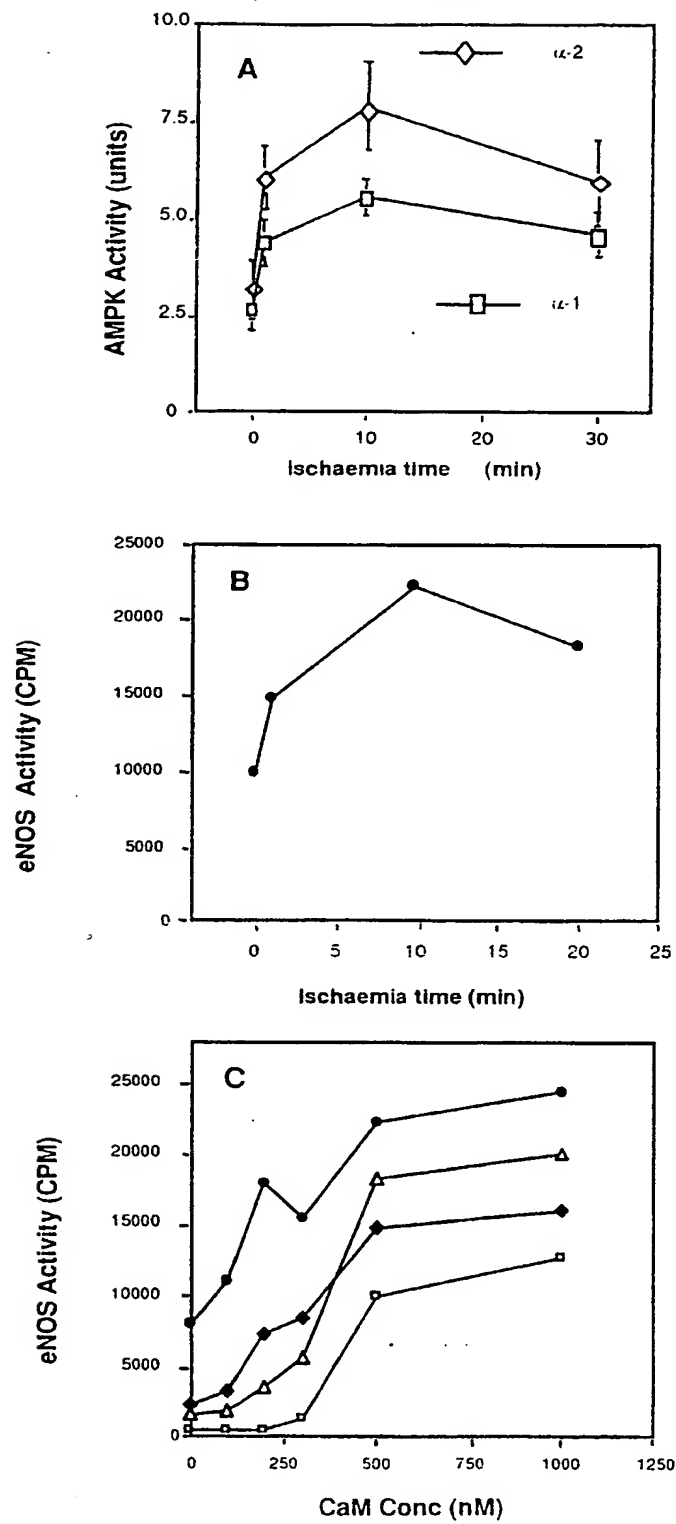


Figure 4

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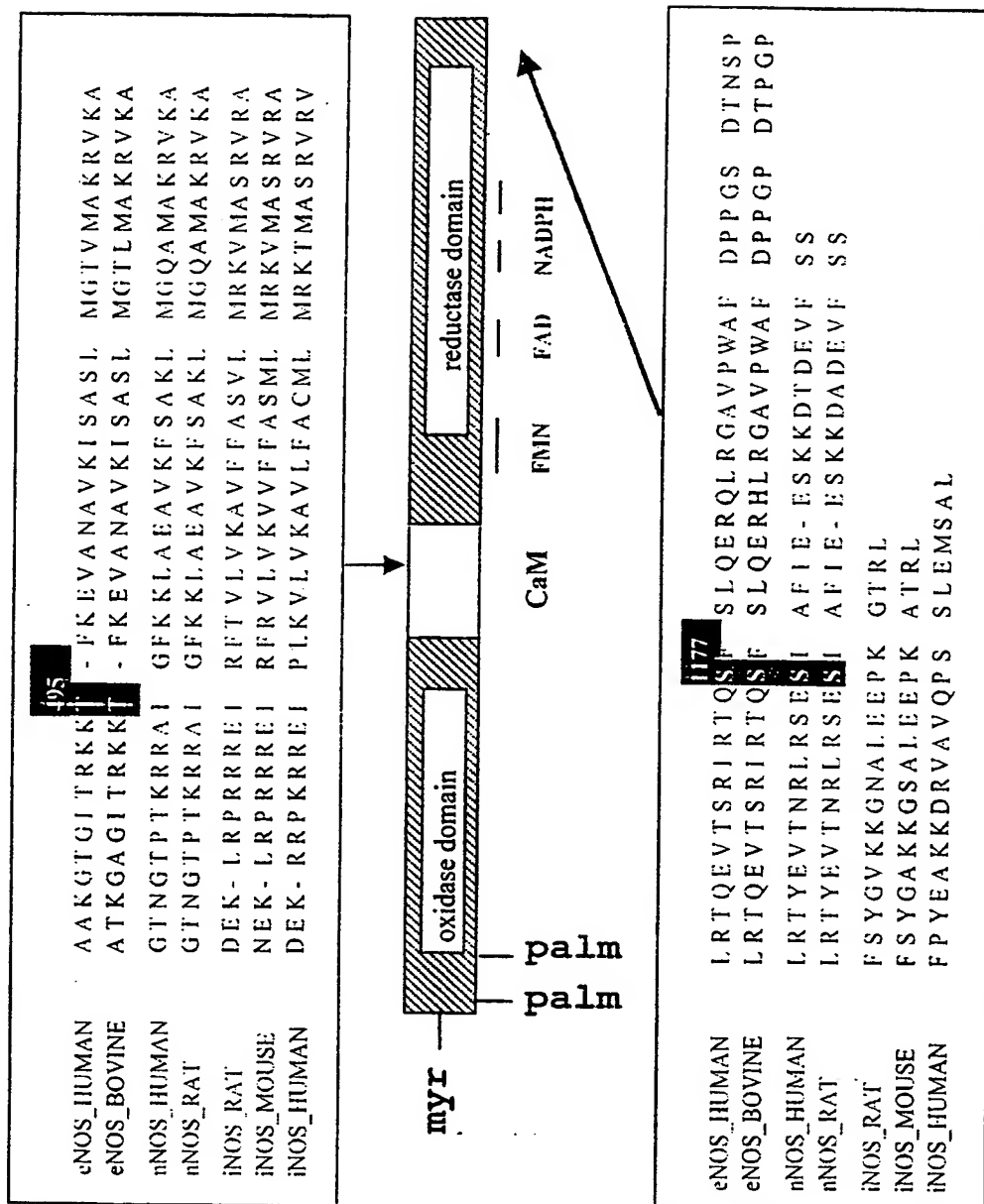
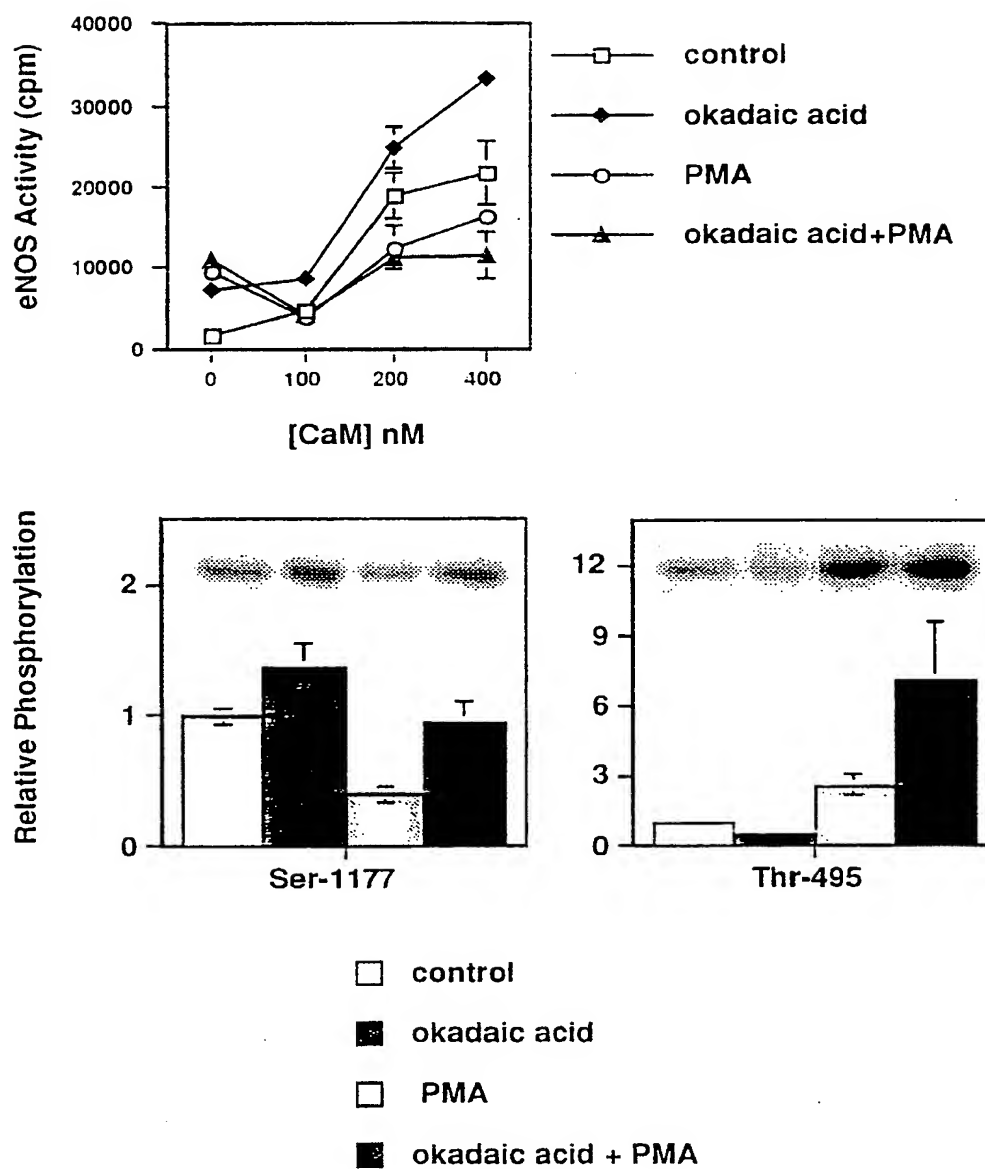
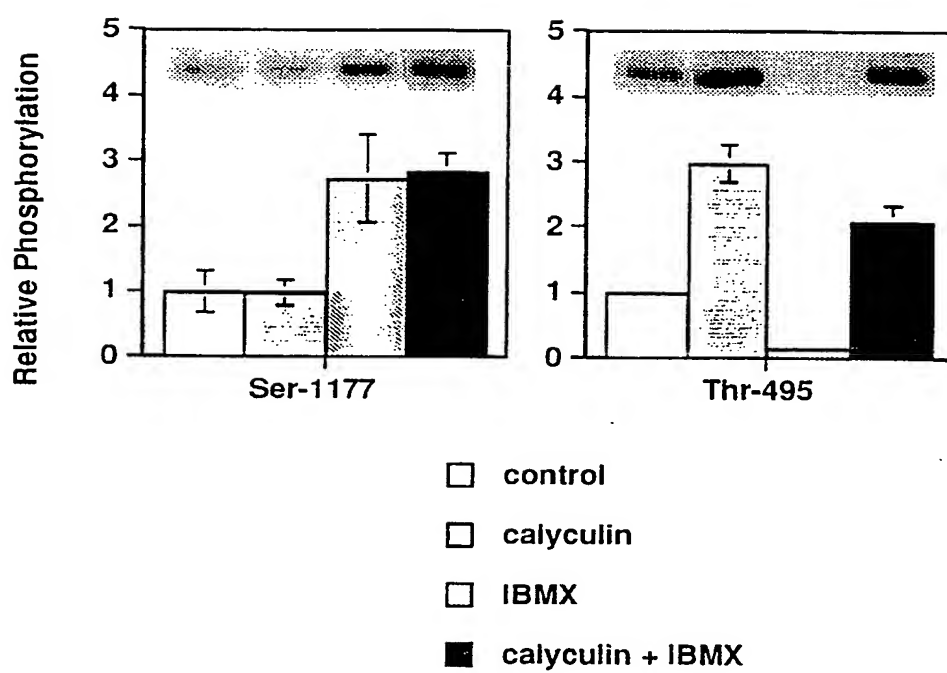


Figure 5

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*Figure 6*

7/9

*Figure 7*

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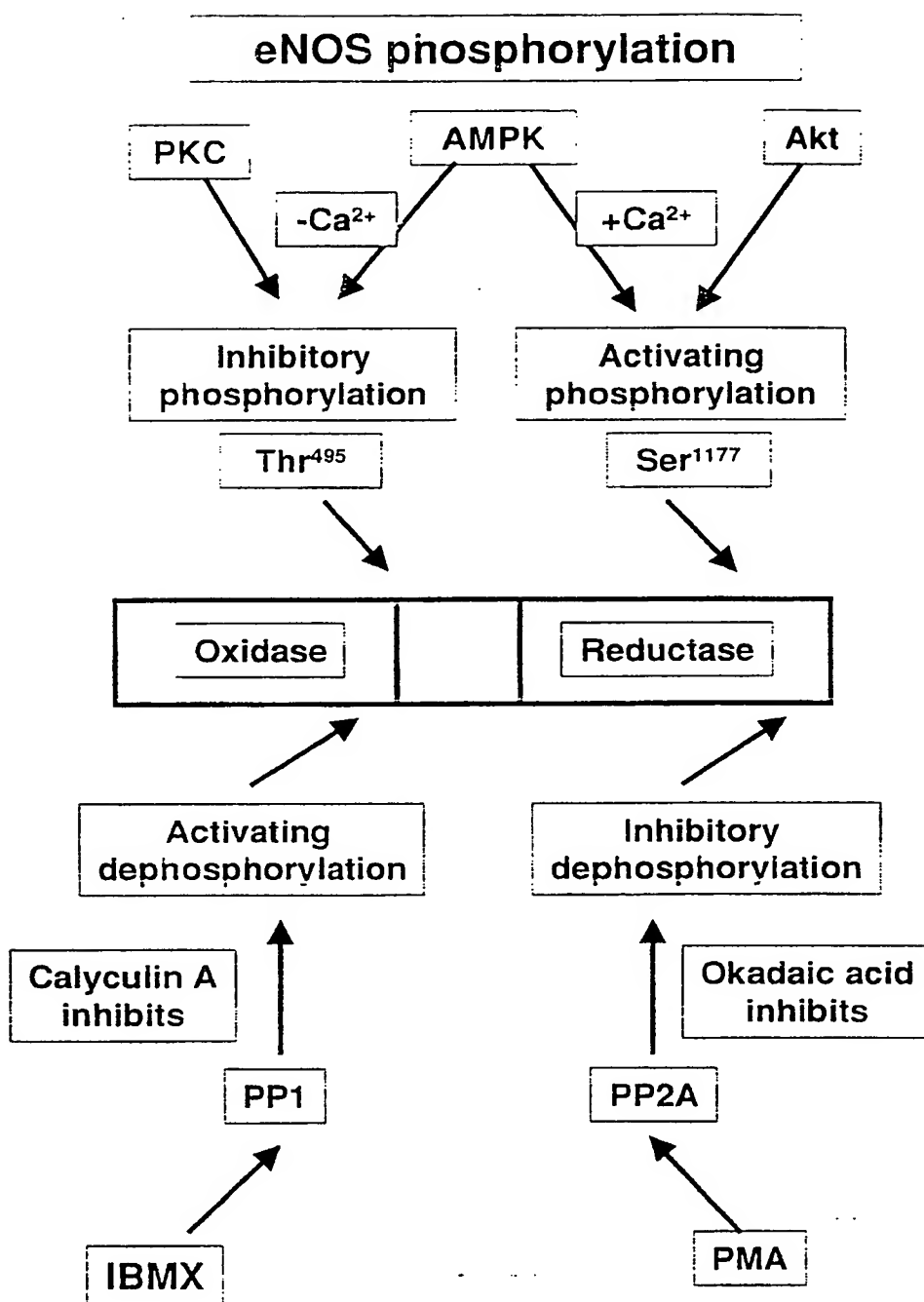


Figure 8

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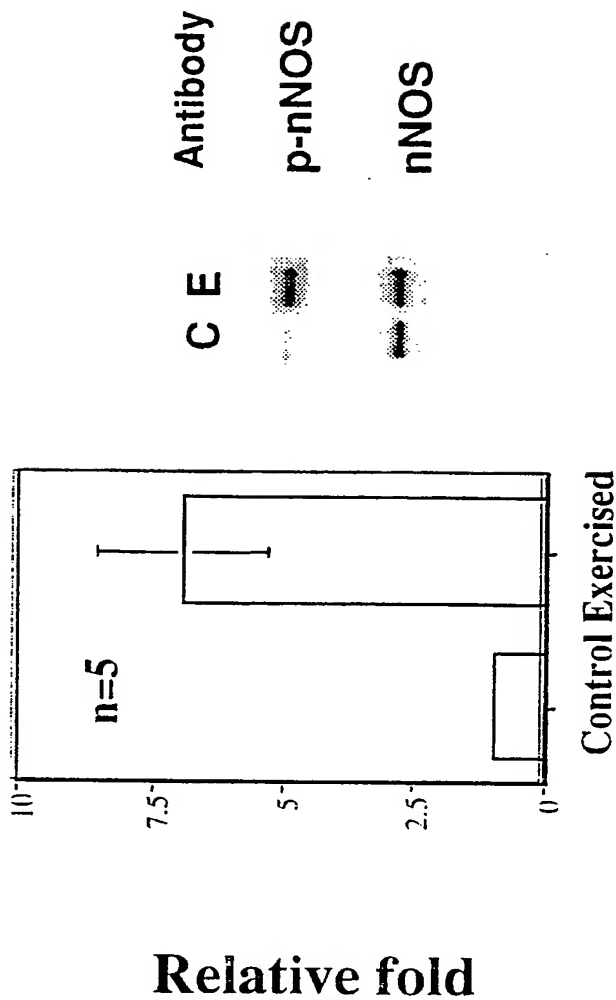


Figure 9



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 99/00968

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int Cl <sup>6</sup> : C12Q 1/26, 1/27, 1/48		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) IPC (WPAT - WORLD PATENTS INDEX), CHEMICAL ABSTRACTS		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched <b>MEDLINE</b>		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, Medline, Chemical Abstracts. Search terms: nitric oxide synthase, ampk, amp protein kinase:		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	FEBS Letters, volume 443, 1999, Chen et al., "AMP-activated protein kinase phosphorylation of endothelial NO synthase", pages 285 to 289	1 to 11
P, X	WO 99/26657 (MEDICAL UNIVERSITY OF SOUTH CAROLINA) 3 June 1999	1, 3, 5 to 9
A	The Journal of Biological Chemistry, volume 267, number 16, 1992, Bredt et al., "Nitric Oxide Synthase Regulatory Sites", pages 10976 to 10981	1 to 1
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